Appendix A

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Improved Glucose and Lipid Metabolism in Genetically Obese Mice Lacking aP2

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ABSTRACT

Adipocyte fatty acid-binding protein, aP2, is a member of the intracellular fatty acid binding protein family. Previously, studies have shown increased insulin sensitivity in aP2-deficient mice with dietary obesity. Here, we asked whether aP2-related alterations in lipolytic response and insulin production are features of obesity-induced insulin resistance and investigated the effects of aP2-deficiency on glucose homeostasis and lipid metabolism in ob/ob mice, a model of extreme obesity. ob/ob mice homozygous for the aP2 null allele (ob/ob-aP2$^{-/-}$) became more obese than ob/ob mice as indicated by significantly increased body weight and fat pad size but unaltered body length. However, despite their extreme adiposity, ob/ob-aP2$^{-/-}$ animals were more insulin-sensitive compared with ob/ob controls, as demonstrated by significantly lower plasma glucose and insulin levels and better performance in both insulin and glucose tolerance tests. These animals also showed improvements in dyslipidemia and had lower plasma triglyceride and cholesterol levels. Lipolytic response to β-adrenergic stimulation and lipolysis-associated insulin secretion was significantly reduced in ob/ob-aP2$^{-/-}$ mice. Interestingly, glucose-stimulated insulin secretion, while virtually abolished in ob/ob controls, was significantly improved in ob/ob-aP2$^{-/-}$ animals. There were no apparent morphological differences in the structure or size of the pancreatic islets between genotypes. Taken together, the data indicate that in obesity, aP2-deficiency not only improves peripheral insulin resistance but also preserves pancreatic β cell function and has beneficial effects on lipid metabolism. (*Endocrinology* **141:** 3388–3396, 2000)

OBESITY, with a prevalence around 55–63% (body mass index ≥ 25 kg/m^2) in the United States population (1, 2), is a major risk factor for the development of type 2 diabetes and associated pathological states such as dyslipidemia, hypertension and atherosclerosis (3, 4). More than 80% of individuals with type 2 diabetes are obese (5). The pathogenesis of type 2 diabetes involves the progressive development of hyperinsulinemia and insulin resistance, a decreased response to insulin in target tissues (6). Another hallmark of the disease is impaired β-cell function that, at least in some cases, is already detectable in stages preceding overt hyperglycemia (7). Although the principal defects of type 2 diabetes are clear, how an expanded fat mass results in any of these pathologies is not well understood.

Possible factors for obesity-induced disorders are lipid molecules that are elevated in obesity, such as long chain nonesterified fatty acids (FFA; FFA). In agreement with a role of systemic FFA in the development of type 2 diabetes, it has been shown that elevation of plasma FFA induces peripheral insulin resistance in humans and rodent models within few hours (8–10). In addition, it has been shown that FFA can have positive or negative effects on insulin secretion, depending on the experimental conditions (11–18). Thus, obesity-induced elevation of systemic FFA might have direct impact on glucose homeostasis via systemic insulin sensitivity and possibly through effects on insulin secretion.

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Adipocyte fatty acid-binding protein, aP2, is a member of the intracellular fatty acid binding protein family (19) highly expressed in adipocytes (20, 21). While the exact cellular functions of aP2 remain largely undefined, our earlier work has indicated an important *in vivo* role for this protein in lipid and glucose metabolism. Mice deficient for aP2 (aP2$^{-/-}$) were shown to be protected from development of insulin resistance and hyperinsulinemia in a high fat diet-induced obesity model, establishing aP2$^{-/-}$ mice as an experimental system to study the pathogenesis of type 2 diabetes (22). In subsequent studies with lean animals, it was shown that the lipolytic response was attenuated in aP2$^{-/-}$ mice and isolated aP2$^{-/-}$ adipocytes (23, 24). Furthermore, lipolysis-associated insulin secretion in the lean aP2$^{-/-}$ mice was profoundly reduced although the pancreatic response to other insulin secretagogues was unaltered (23). Although the mechanisms underlying these changes are not yet clear, the interesting possibility was raised that an intrinsically reduced propensity to secrete insulin in response to lipid or other meditors might contribute to the protection of aP2$^{-/-}$ mice from hyperinsulinemia and consequently insulin resistance.

To test the relevance of these observations in obesity, in a uniform genetic setting, we generated ob/ob mice lacking aP2. In these animals, parameters relevant to obesity, insulin resistance, insulin secretion, and plasma lipids were studied longitudinally to assess the overall effect of aP2-deficiency on obesity-related metabolic abnormalities. Lipolysis, lipolysis-associated insulin secretion, and β cell function were studied in more detail in a subset of animals. Our results demonstrate that aP2-deficiency has significant beneficial effects on

insulin resistance in the ob/ob model, and that the lack of aP2 results in reduced lipolysis and lipolysis-induced insulin secretion in obesity.

Materials and Methods

Generation of ob/ob and ob/ob-aP2$^{-/-}$ mice

Mice deficient in aP2 (backcrossed 12 times into C57BL/6 background) were intercrossed with animals heterozygote in the ob (leptin) locus (OB/ob C57BL/6) to generate double heterozygotes (OB/ob-aP2$^{+/-}$). These mice were then intercrossed to generate OB/ob-aP2$^{-/-}$ or OB/ob-aP2$^{+/+}$ mice, which subsequently acted as parents to lean and obese (OB/OB and ob/ob respectively) animals either wild-type (aP2$^{+/+}$) or null (aP2$^{-/-}$) in the aP2 locus. All mice that are homozygous for the ob mutation (ob/ob) developed obesity and hence, are referred to as obese in the text. Mice were kept on a 12-h light cycle, staying in dark between 1900 h and 0700 h and were fed either a standard mouse chow or a high-fat, high-carbohydrate diet *ad libitum* (Diet F3283, Bio-Serv, Frenchtown, NJ). Experiments and sample collection took place in the early afternoon after either a 6-hour daytime food withdrawal for steady state measurements or following a 24-h fast.

Metabolic measurements

Total body weights were measured monthly from age 4–16 weeks. Blood samples were collected at 4, 8, and 12 weeks of age. Glucose concentrations in plasma were measured using glucose analyzer glucose strips (Medisense, Bedford, MA). Serum insulin and C-peptide were measured with a monoclonal insulin RIA (Linco Research, Inc., St. Louis, MO). Glucose and insulin tolerance tests were performed on conscious male animals following a 24 h fast by ip administration of glucose (1.8 mg/g) and measurements of tail blood glucose at 15, 30, 45, 60, 90, and 120 min. The insulin tolerance test was done similarly except for the injection of recombinant mouse insulin (1 U/kg, Sigma, St. Louis, MO) and an additional blood glucose measurement at 150 min. Plasma tri-glycerides, glycerol, cholesterol, and FFA levels were measured using commercially available color enzymatic assays (Sigma and Wako Pure Chemical Industries Ltd., Richmond, VA). In both CL 316,243- (β3-adrenoreceptor specific agonist provided by Dr. K. Steiner, Wyeth-Ayerst Laboratories, Inc., Princeton, NJ) stimulated lipolysis and glucose-stimulated insulin secretion experiments, the animals were fasted 24 h before the experiments. The compounds were dissolved in PBS and injected ip at a dose of 0.1 mg/kg (CL 316,243) and 1.8 mg/g (glucose). In all experiments, except the tolerance tests, blood was collected from the orbital plexus after anesthetizing animals with methoxyflurane (Mallinckrodt, Inc. Veterinary). Dynamic experiments were performed in males, whereas steady-state measurements were in both males and females. For all measurements, such as tolerance tests, CL 316,243 stimulated lipolysis, or glucose-stimulated insulin secretion, statistical analysis was performed using ANOVA repeated measurements.

Staining of pancreatic sections

Following administration of an overdose of sodium amytal, pancreas was excised from each animal *in toto*. After excision, each pancreas was lightly blotted, weighed, fixed in Bouin's fixative, and embedded in paraffin by routine techniques. Sections were immunostained (immunoperoxidase) to examine the β cells using antibodies against insulin (guinea pig antiporcine insulin, Linco Research, Inc., St. Charles, MO, 1:200) or the non-β-endocrine cells of the islet (the mantle) using a cocktail of antibodies against glucagon (antibovine final dilution 1:3000, gift of Dr. M. Appel), somatostatin (rabbit antisynthetic final dilution 1:300, made in the laboratory of Dr. S. Bonner-Weir), and pancreatic polypeptide (rabbit antibovine final dilution 1:3000, gift of Dr. R. Chance, Eli Lilly & Co., Indianapolis, IN). The sections were incubated overnight at 4 C, washed in PBS, incubated with goat antirabbit IgG as a secondary antibody, washed with Tris buffer (pH 7.4), incubated with a peroxidase antirabbit serum (Cappel Laboratories, Cochranville, PA), stained with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Immunochemicals) and counterstained with hematoxylin. Insulin staining was done similarly except for the use of a goat anti guinea pig secondary antibody. The slides were evaluated blindly to the genotype of the animals.

Results

Body weight and adiposity of mice

To assess the potential effects of aP2-deficiency on body weight regulation and energy metabolism, we monitored growth and total body weight of lean and obese aP2$^{+/+}$ and aP2$^{-/-}$ animals of both sexes for a period of 16 weeks. As shown in Fig. 1, no significant difference in body weight was observed between lean aP2-deficient and wild-type animals. However, throughout the study period, ob/ob mice deficient in aP2 (ob/ob-aP2$^{-/-}$) displayed increased obesity compared with obese control animals (ob/ob). At 16 weeks of age both male and female ob/ob-aP2$^{-/-}$ mice weighed 15% more compared with ob/ob animals ($P < 0.001$).

To determine if the increased body weight in ob/ob-aP2$^{-/-}$ mice was the result of increased axial growth, we measured the body length of these animals. No difference

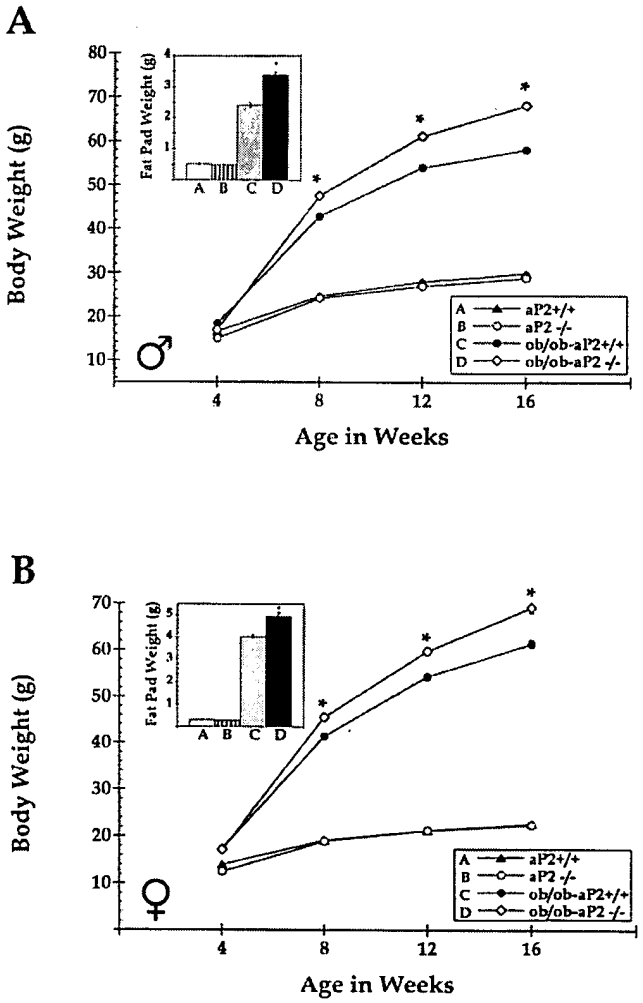

FIG. 1. Growth curves and fat pad weights of lean and obese aP2$^{+/+}$ and aP2$^{-/-}$ mice. Data from male (A) and female (B) mice are shown as mean ± SE (n = 27–36). * indicates $P < 0.05$ ob/ob-aP2$^{-/-}$ *vs.* ob/ob-aP2$^{+/+}$.

was evident in the nasal-anal length of the $aP2^{-/-}$ and $aP2^{+/+}$ animals in either lean or obese group (data not shown). Epididymal or periovarian fat pad weight of each animal was also weighed to determine if a parallel increase is evident in adiposity (Fig. 1). No significant difference in fat pad weight was observed between lean $aP2^{-/-}$ and $aP2^{+/+}$ animals in both sexes. However, the fat pad weights of ob/ob-$aP2^{-/-}$ mice of both sexes were significantly higher compared with ob/ob controls, suggesting increased adiposity in these animals (3.4 ± 0.08 *vs.* 2.4 ± 0.08 g in males and 4.9 ± 0.12 *vs.* 4.0 ± 0.08 g in females, $P < 0.001$).

Glucose homeostasis

To determine the effects of the absence of aP2 on glucose metabolism, we measured glucose and insulin levels in both steady (after 6-h day time food withdrawal) and fasted (24 h) states. The lean animals remained euglycemic throughout the study, and there was no significant difference in blood glucose between lean $aP2^{-/-}$ and $aP2^{+/+}$ mice in either condition (Table 1). Similarly, the plasma insulin levels of all lean mice were within normal boundaries in the fed state. In the fasted state, insulin levels of male $aP2^{-/-}$ mice were higher than that of the $aP2^{+/+}$ animals, but this difference did not reach statistical significance in the females. In all obese groups, the animals developed hyperglycemia and hyperinsulinemia compared with the lean controls. However, both conditions were significantly improved in the $aP2^{-/-}$ animals and in both sexes, ob/ob-$aP2^{-/-}$ displayed lower plasma glucose and insulin levels, indicating a better glucose homeostasis. Overall, plasma glucose levels in ob/ob-$aP2^{-/-}$ mice were decreased by 20–25% in both sexes compared with ob/ob controls ($P < 0.001$). Similarly, a 35–40% reduction in plasma insulin levels was evident in ob/ob-$aP2^{-/-}$ in both conditions and in both sexes.

To determine insulin sensitivity directly in lean and obese $aP2^{-/-}$ mice and controls, ip insulin and glucose tolerance tests were performed (Fig. 2). The deficiency of aP2 had no effect on insulin sensitivity in the lean groups. However, in obese animals, the ob/ob-$aP2^{-/-}$ mice displayed a signifi-

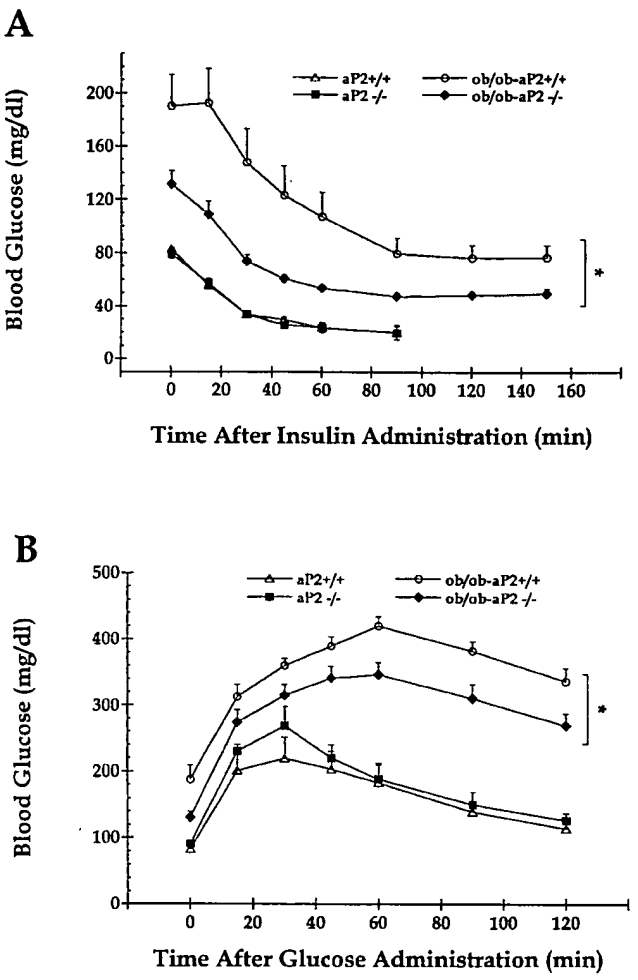

FIG. 2. Insulin (A) and glucose (B) tolerance tests in lean and obese $aP2^{+/+}$ and $aP2^{-/-}$ mice. n = 4, 4, 13, 19 for $aP2^{+/+}$, $aP2^{-/-}$, ob/ob-$aP2^{+/+}$ and ob/ob-$aP2^{-/-}$, respectively. Data are shown as mean ± SE. * indicates $P < 0.05$ ob/ob-$aP2^{-/-}$ *vs.* ob/ob-$aP2^{+/+}$.

TABLE 1. Plasma measurements of metabolic parameters in lean and obese $aP2^{+/+}$ and $aP2^{-/-}$ mice at 12 weeks of age

	$aP2^{+/+}$		$aP2^{-/-}$		ob/ob-$aP2^{+/+}$		ob/ob-$aP2^{-/-}$	
	Steady	Fasted	Steady	Fasted	Steady	Fasted	Steady	Fasted
Male								
Glucose (mg/dl)	155 ± 7	73 ± 2	149 ± 2	79 ± 3	290 ± 10	159 ± 11	237 ± 8[b]	113 ± 4[b]
Insulin (ng/ml)	1.8 ± 0.2	0.26 ± 0.03	2.1 ± 0.2	0.56 ± 0.14[a]	10.5 ± 0.5	3.3 ± 0.2	6.7 ± 0.5[b]	2.30 ± 0.1[b]
FFA (mM)	0.45 ± 0.01	1.01 ± 0.03	0.50 ± 0.02	1.12 ± 0.02[a]	0.48 ± 0.01	0.64 ± 0.04	0.55 ± 0.02[b]	0.87 ± 0.02[b]
Glycerol (mM)	0.20 ± 0.01	0.45 ± 0.01	0.18 ± 0.01[a]	0.44 ± 0.01	0.28 ± 0.01	0.38 ± 0.03	0.23 ± 0.01[b]	0.43 ± 0.01
Triglyceride (mg/dl)	46 ± 1	80 ± 2	44 ± 1	69 ± 3[a]	63 ± 3	71 ± 5	48 ± 2[b]	68 ± 3
Cholesterol (mg/dl)	88 ± 1	89 ± 2	91 ± 2	90 ± 2	183 ± 4	156 ± 6	175 ± 4	150 ± 8
Female								
Glucose (mg/dl)	140 ± 5	66 ± 1	134 ± 2	66 ± 3	252 ± 14	174 ± 16	209 ± 9[b]	125 ± 5[b]
Insulin (ng/ml)	2.0 ± 0.2	0.32 ± 0.10	2.2 ± 0.3	1.0 ± 0.2	11.1 ± 0.8	3.4 ± 0.9	6.9 ± 0.8[b]	2.1 ± 0.3[b]
FFA (mM)	0.50 ± 0.03	1.07 ± 0.05	0.50 ± 0.04	1.23 ± 0.04[a]	0.51 ± 0.02	0.71 ± 0.07	0.62 ± 0.04[b]	0.99 ± 0.06[b]
Glycerol (mM)	0.23 ± 0.01	0.50 ± 0.02	0.19 ± 0.01[a]	0.49 ± 0.01	0.28 ± 0.01	0.42 ± 0.01	0.23 ± 0.01[b]	0.47 ± 0.03
Triglyceride (mg/dl)	40 ± 1	72 ± 6	37 ± 2	53 ± 1[a]	47 ± 1	47 ± 2	41 ± 1[b]	50 ± 1
Cholesterol (mg/dl)	85 ± 1	88 ± 2	83 ± 2	91 ± 3	173 ± 4	163 ± 6	162 ± 3[b]	142 ± 2[b]

In the steady-state, numbers were n = 27–36 for male and n = 27–31 for female animals. In the fasted state, numbers were n = 17–38 for male and n = 6–19 for female animals.

[a] indicates $p < 0.05$ when $aP2^{-/-}$ is compared with $aP2^{+/+}$ mice.

[b] indicates $p < 0.05$ when ob/ob-$aP2^{-/-}$ is compared with ob/ob-$aP2^{+/+}$ mice.

cantly stronger hypoglycemic response to a given dose of insulin compared with ob/ob controls, which is consistent with increased insulin sensitivity. Similarly, following the administration of glucose, ob/ob-aP2$^{-/-}$ animals displayed improved glucose profiles consistent with increased insulin sensitivity compared with the ob/ob animals (Fig. 2).

Plasma cholesterol and triglyceride

The fact that increased plasma levels of triglyceride and cholesterol are important risk factors for atherosclerosis and are frequently associated with insulin resistance (3) prompted us to determine the plasma concentrations of triglycerides and cholesterol in lean and obese aP2$^{-/-}$ and control mice. In the lean animals, we did not observe an effect of aP2-deficiency on plasma cholesterol levels (Table 1). However, female ob/ob-aP2$^{-/-}$ mice had lower plasma cholesterol levels compared with ob/ob controls (10–15% decrease, $P < 0.01$). This difference was only 5% in males and was not statistically significant. aP2 deficiency also had moderate effects in reducing plasma triglyceride levels, under some but not all conditions. In the lean group, plasma triglyceride levels were decreased 15% in males and 26% in females ($P < 0.001$) in fasted animals but no significant difference was observed in the steady-state (Table 1). In contrast, ob/ob-aP2$^{-/-}$ mice of both sexes had significantly lower steady state plasma triglyceride levels (25% in males and 10% in females, $P < 0.01$) compared with ob/ob controls.

Plasma glycerol and FFA

Increased FFAs are associated with obesity and have been postulated to be causally involved in the development of hyperinsulinemia and insulin resistance (10, 25, 26). To test if systemic FFA levels play a role in the observed improvement in insulin sensitivity in ob/ob-aP2$^{-/-}$ mice, we measured plasma levels of FFA and glycerol, the two products of lipolysis, under both basal and CL 316,243-stimulated conditions. Baseline plasma glycerol levels were lower in aP2$^{-/-}$ lean and obese animals of both sexes (Table 1). This decrease was 12–18% in the lean and 15% in the obese groups ($P < 0.04$). In the fasted state the glycerol levels increased in all mice and no difference was evident between aP2$^{-/-}$ and aP2$^{+/+}$ animals. In general, a small (10%, $P < 0.05$), but consistent increase was observed in the plasma FFA levels of aP2$^{-/-}$ mice with the exception of lean animals in the steady-state (Table 1). In the obese group, the aP2-deficient mice also had higher plasma FFA levels (14–20% in the steady state and 26–29% after fasting, $P < 0.01$).

Lipolysis

Lipolysis is an adipocyte function that might contribute to the development of insulin resistance in obesity (27, 28). Thus, we examined *in vivo* lipolysis stimulated by CL-316,243 (0.1 mg/kg), a specific agonist of the β3-adrenergic receptor (β3-AR) (29), which is primarily expressed in adipose tissue and gastrointestinal tract (30, 31). For these experiments, blood samples were collected before and 8, 16, 30 and 60 min after the ip injection of 0.1 mg/kg CL 316,243. While plasma glycerol levels increased over basal levels in all animals, the extent of this increase was significantly smaller in both lean and obese aP2-deficient mice (Fig. 3A). At 8 min, when the response is maximal, the rise in plasma glycerol levels was 2.2-fold in ob/ob-aP2$^{-/-}$ mice compared with the 2.8-fold increase in ob/ob-aP2$^{+/+}$ controls ($P < 0.05$). Similar to glycerol, FFA levels also increased in all animals following CL 316,243 administration reaching maximum levels at 30 min (Fig. 3B). This increase, however, was much smaller in both lean and obese aP2-deficient animals throughout the experiment. At 30 min, the plasma FFA levels of ob/ob-aP2$^{-/-}$ animals increased by only 1.8-fold, whereas a 2.8-fold increase was evident in ob/ob controls indicating a reduced lipolytic response associated with aP2-deficiency.

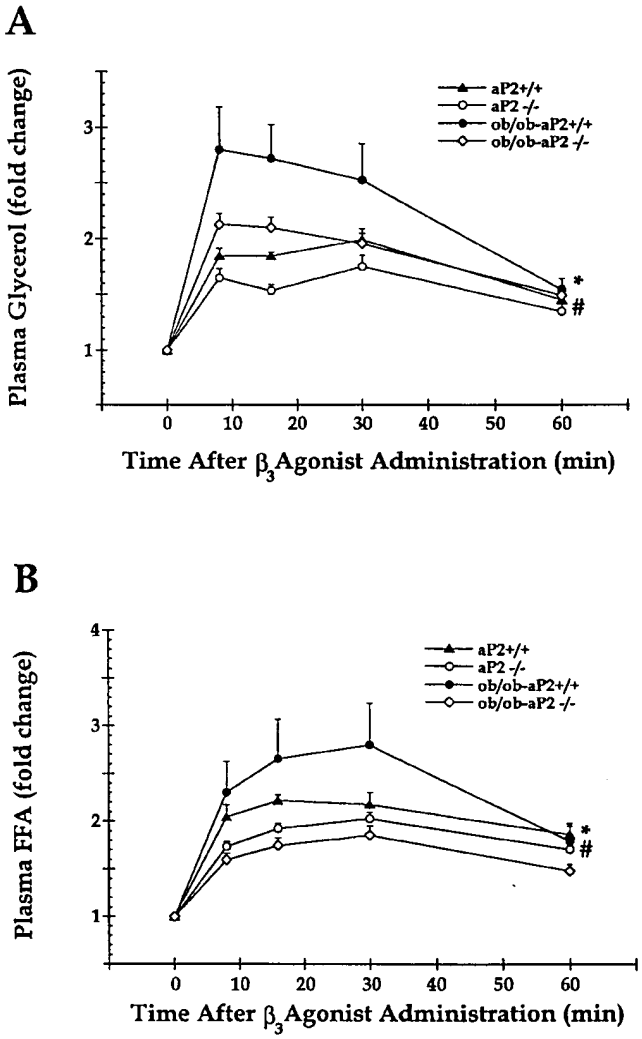

FIG. 3. Lipolysis in lean and obese aP2$^{+/+}$ and aP2$^{-/-}$ mice. Plasma glycerol (A) and FFA (B) were measured after administration of β3-adrenergic receptor agonist. Data are shown as mean ± SE (n = 10–20). * indicates $P < 0.05$ ob/ob-aP2$^{-/-}$ *vs.* ob/ob-aP2$^{+/+}$. # indicates $P < 0.05$ aP2$^{-/-}$ *vs.* aP2$^{+/+}$. Basal plasma glycerol levels were 0.337 ± 0.02, 0.40 ± 0.03, 0.34 ± 0.04 and 0.42 ± 0.02 mM for aP2$^{+/+}$, aP2$^{-/-}$, ob/ob-aP2$^{+/+}$ and ob/ob-aP2$^{-/-}$, respectively. Basal plasma FFA levels were 0.92 ± 0.05, 1.03 ± 0.03, 0.59 ± 0.05, and 0.86 ± 0.03 mM for aP2$^{+/+}$, aP2$^{-/-}$, ob/ob-aP2$^{+/+}$ and ob/ob-aP2$^{-/-}$, respectively.

Lipolysis-induced insulin secretion

Stimulation of adipocyte lipolysis *in vivo* is associated with increased insulin secretion (32, 33). Because alterations in this response could be related to the observed reduction of hyperinsulinemia in ob/ob-aP2$^{-/-}$ animals, we studied β-AR stimulated insulin secretion in lean and obese mice. For these experiments, blood samples were collected before and 8, 16, 30, and 60 min after ip administration of 0.1 mg/kg CL 316,243. Interestingly, in both lean and obese groups, we observed a dramatic reduction in the β3-AR-stimulated insulin secretion in aP2$^{-/-}$ mice compared with aP2$^{+/+}$ controls (Fig. 4A). In the lean group, the plasma insulin levels started to increase by 8 min and reached a maximum at 30 min. At this time, the plasma insulin levels were increased by 24- and 52-fold in aP2$^{-/-}$ and aP2$^{+/+}$ mice, respectively. This difference between the lean groups subsided by 60 min. In the obese groups, a notable rise in circulating insulin levels was not detected until 30 min after stimulation. The increase in plasma insulin levels in the ob/ob-aP2$^{-/-}$ (2- and 4-fold) was significantly smaller than ob/ob controls (4- and 6-fold) at 30 and 60 min, respectively. These data demonstrate that the insulin secretory response to lipolytic stimulation is strikingly reduced in aP2-deficient animals.

β-cell function and morphology

Because we observed reduced β3-AR-mediated insulin secretion in both lean and obese aP2$^{-/-}$ mice, we asked whether this difference is the result of generally reduced β cell function or a specific unresponsiveness to only lipolytic stimuli. Hence, we examined the insulin secretion response to glucose in the obese and lean groups. In the lean animals, the magnitude of glucose-stimulated insulin and C-peptide secretion was not different between the aP2$^{-/-}$ mice and aP2$^{+/+}$ controls (Fig. 4, B and C). In the obese group, the overall response to glucose was not robust. Interestingly, aP2-deficiency rendered ob/ob mice more responsive to glucose, reflected in higher plasma insulin and C-peptide concentrations following glucose administration compared with control animals. One hour after glucose injection, plasma insulin levels of ob/ob-aP2$^{-/-}$ mice was increased by 2.5-fold, whereas there was almost no change in plasma insulin levels of the ob/ob mice. Similarly, C-peptide levels increased 2-fold in ob/ob-aP2$^{-/-}$ mice, whereas no response to glucose was evident in the ob/ob animals.

Finally, to determine whether any of the observed differences in insulin secretion were due to differences in pancreatic islet size or composition, we examined the pancreas of lean and obese aP2-deficient animals by immunohistochemistry. Islet morphology (size, shape, and organization of the non-β-cell mantle) was evaluated in blind fashion. Genotypes could not be separated on the basis of differences in pancreatic morphology. There was no significant difference in the pancreatic morphology or weight between wild-type and aP2-deficient mice (Fig. 5). No significant differences were also evident in the extent of islet degranulation in aP2$^{-/-}$ and aP2$^{+/+}$ obese animals compared with their lean counterparts. In addition, the obese animals, regardless of their genotype, had similar amounts of fat droplets scattered between the lobes and in the exocrine portion of pancreas (see Fig. 5).

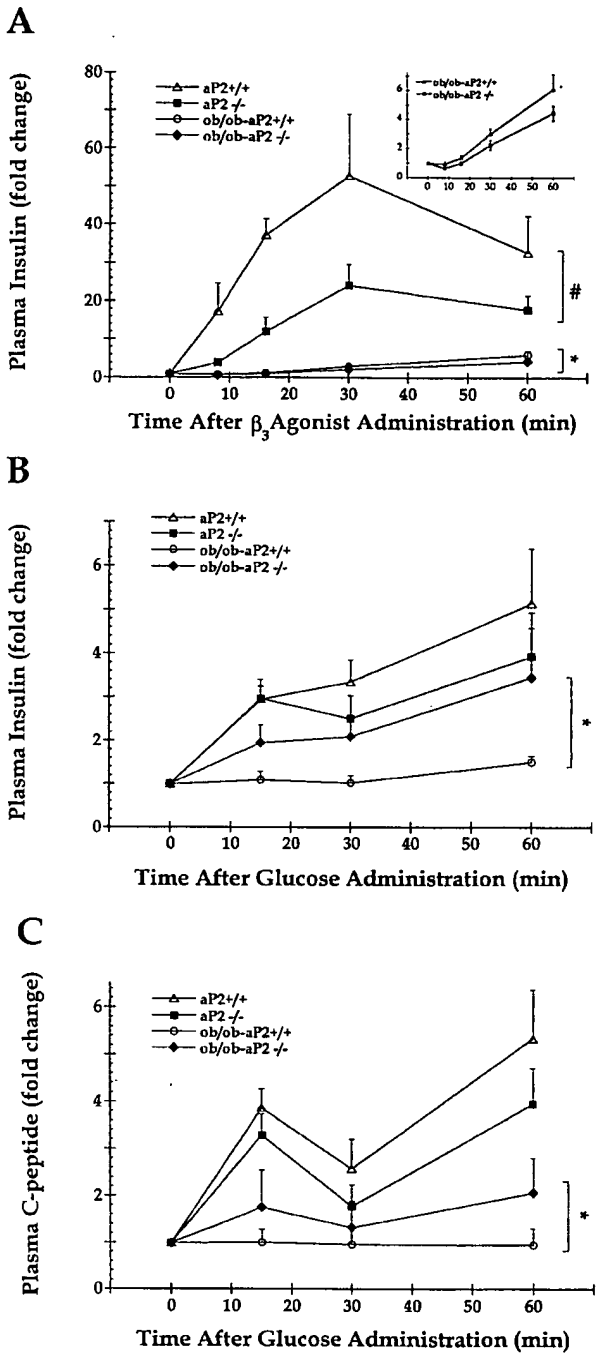

FIG. 4. Insulin secretion in lean and obese aP2$^{+/+}$ and aP2$^{-/-}$ mice. Plasma insulin levels were measured after administration of β3-adrenergic receptor agonist (A) or glucose (B). We also measured plasma C-peptide levels after administration of glucose (C). Data are shown as mean ± SE (n = 9–20). * indicates $P < 0.05$ ob/ob-aP2$^{-/-}$ *vs.* ob/ob-aP2$^{+/+}$. # indicates $P < 0.05$ aP2$^{-/-}$ *vs.* aP2$^{+/+}$. In (A), the basal plasma insulin levels were 0.14 ± 0.01, 0.20 ± 0.01, 3.70 ± 0.41, and 2.41 ± 0.30 ng/ml for aP2$^{+/+}$, aP2$^{-/-}$, ob/ob-aP2$^{+/+}$ and ob/ob-aP2$^{-/-}$, respectively. In (B), the basal plasma insulin levels were 0.39 ± 0.02, 0.40 ± 0.02, 2.2 ± 0.2 and 1.4 ± 0.1 ng/ml for aP2$^{+/+}$, aP2$^{-/-}$, ob/ob-aP2$^{+/+}$ and ob/ob-aP2$^{-/-}$, respectively. Basal plasma C-peptide levels were 165 ± 20, 228 ± 35, 840 ± 68 and 816 ± 56 pM for aP2$^{+/+}$, aP2$^{-/-}$, ob/ob-aP2$^{+/+}$, and ob/ob-aP2$^{-/-}$, respectively.

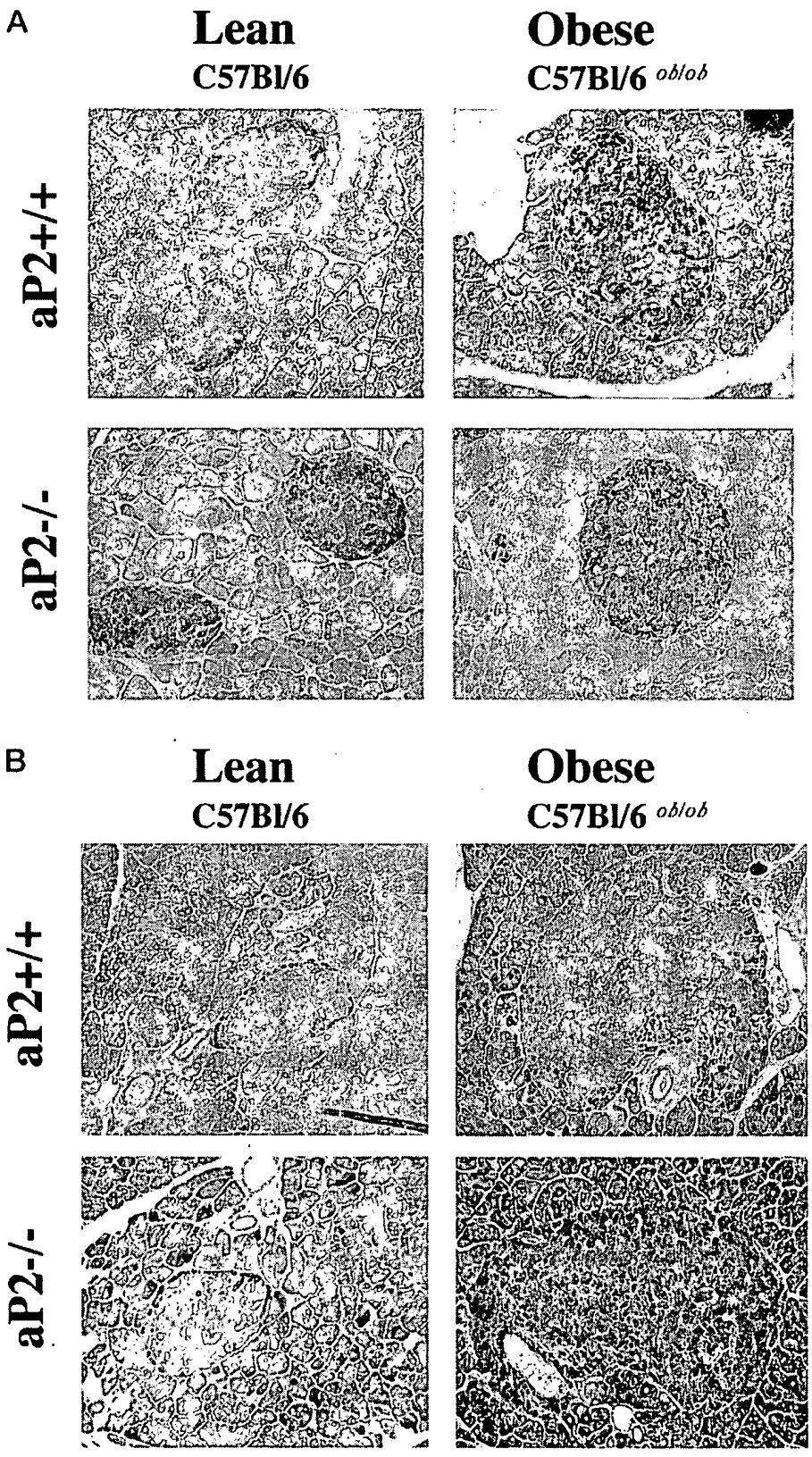

FIG. 5. Islet size and morphology in lean and obese $aP2^{+/+}$ and $aP2^{-/-}$ mice. Immunostaining of pancreatic sections were carried out using antibodies to insulin (A) and non-β-cell hormones (B). Representative sections are shown from all groups studied (n = 4–6).

Discussion

Obesity is ubiquitously associated with insulin resistance. More than 80% of the individuals with type 2 diabetes are also obese and an overwhelming majority of obese individuals express abnormalities of various degrees in insulin action and glucose metabolism. While the close relationship between obesity and diabetes has long been known, understanding the molecular mechanisms by which excess adiposity leads to this condition has been difficult.

Much attention has been placed on muscle tissue in systemic insulin resistance, both due to its mass and the fact that defective insulin action is detectable at this site early in the development of type 2 diabetes. Interestingly, recent studies with genetic rodent models have put unexpected weight on the role of adipose tissue and pancreatic islets as dominant sites impacting systemic insulin resistance and development of type 2 diabetes (34–39). In line with this notion are findings in mice genetically deficient in aP2, the adipocyte fatty acid-binding protein. The expression of aP2 is highly restricted to differentiated adipocytes (20, 21) and recently it has been detected in activated macrophages (Ref. 40 and our unpublished results). In a dietary model of obesity, aP2-deficiency leads to substantially increased insulin sensitivity (22), demonstrating that an isolated defect in the lipid biology of adipocytes might have a significant systemic effect on the course of obesity-induced hyperinsulinemia and insulin resistance.

How could an alteration in adipose lipid metabolism effect systemic glucose metabolism? A potential scenario is that expansion of fat tissue and concomitant insulin resistance of adipocytes will lead to increased lipolysis and release of byproducts such as FFA and cytokines from adipose tissue, eventually resulting in reduced glucose disposal and increased hepatic glucose production. This then stimulates the secretion of insulin to compensate for insulin resistance. Hyperinsulinemia further promotes insulin resistance at target sites through receptor desensitization and, indirectly, through its effects on lipogenesis. This vicious cycle will eventually result in chronic hyperglycemia, defective glucose-stimulated insulin secretion, β-cell pathologies and development of frank diabetes. According to this model, one potential strategy to disturb this vicious cycle will be to enhance insulin action in adipose tissue and prevent excess adipose tissue lipolysis. If the output of the adipose tissue is a critical and early stimulus for hyperinsulinemia in obesity, then decreased lipolysis and the associated products should be associated with lower rates of insulin secretion, and preservation of the β cell response to glucose.

Interestingly, in studies using lean animals, our group as well as Coe *et al.*, have recently demonstrated decreased lipolysis in $aP2^{-/-}$ adipocytes *in vitro* and $aP2^{-/-}$ mice *in vivo* (23, 24). The molecular mechanisms underlying impaired lipolysis in the absence of aP2 are not yet clear. In adipose tissue of aP2-deficient mice, there does not appear to be any quantitative defect in the major components of the lipolytic machinery. Interestingly, recent studies demonstrated that aP2 directly interacts with hormone sensitive lipase (HSL), raising the possibility that this interaction is critical in the efficiency of this enzyme (41).

During studies on lipolysis, we also made the intriguing observation that lipolysis-associated insulin secretion was dramatically reduced in lean $aP2^{-/-}$ mice while the response to other insulin secretagogues remained intact (23). These observations prompted us to postulate that the decreased lipolysis and the subsequent insulin secretory response to the lipolytic products of the adipocyte might underlie the improved overall glucose homeostasis in obese $aP2^{-/-}$ animals. To directly test this hypothesis, we have generated genetically obese mice by intercrossing $aP2^{-/-}$ animals with the ob/ob mice, to establish a well-defined obesity model with aP2-deficiency.

In support of earlier observations in diet-induced obesity, this study clearly demonstrated that aP2-deficiency is associated with significantly improved glucose and lipid metabolism even in the presence of extreme obesity resulting from leptin deficiency. Although ob/ob-$aP2^{-/-}$ mice were even heavier than the ob/ob-$aP2^{+/+}$ animals, they displayed a much improved glucose metabolism compared with the obese controls. This was manifested in significantly lower plasma glucose and insulin levels throughout the experimental period and by their better performance in insulin and glucose tolerance tests. The insulin-sensitizing effect of aP2-deficiency was limited to the obese state, as we did not observe a trend toward higher insulin sensitivity in lean $aP2^{-/-}$ animals. These improvements were, however, not complete since the ob/ob-$aP2^{-/-}$ mice still displayed insulin resistance compared with lean animals.

Next, we tested whether aP2-deficiency leads to alterations in lipolysis and β cell responses in the presence of severe obesity. First, we demonstrated that the lipolytic response to β3-adrenergic receptor stimulation is significantly decreased in lean and, even more so, in the obese $aP2^{-/-}$ mice compared with control animals. This was shown by a reduction in the levels of both glycerol and FFA levels upon β3-AR stimulation in $aP2^{-/-}$ and ob/ob $aP2^{-/-}$ mice. In our previous studies, we have observed significant alterations in glycerol but not in FFA levels in lean animals upon lipolytic stimuli. The extent of this response appears to be related to the metabolic state of the animals because, as shown here, 24 h fasting led to decreased responses in both glycerol and FFA, whereas our previous experiments, performed in the day time resting state, led only to decreased glycerol response (23).

Second, we demonstrate that in both lean and obese mice the lipolysis-associated increase in plasma insulin was substantially reduced in $aP2^{-/-}$ animals compared with their respective controls following β3-adrenergic stimulation. Similar results were also observed in $aP2^{-/-}$ mice made obese by a high fat diet (data not shown). These alterations were not related to a pancreatic defect or general unresponsiveness of the islets. No significant alterations were evident in the morphology of the pancreatic β cells between $aP2^{-/-}$ and $aP2^{+/+}$ animals. The glucose-stimulated insulin secretion was normal and comparable to the lean mice. Most strikingly, glucose-stimulated insulin secretion was partially preserved in ob/ob $aP2^{-/-}$ mice, whereas it was completely abolished in ob/ob controls. These data are consistent with the increased glucose tolerance in these animals and show that the beneficial effect of aP2 deficiency is not only

manifested in enhanced peripheral insulin sensitivity but also in better maintained pancreatic β cell function, both of which potentially contribute to improved glucose metabolism.

The mechanisms by which aP2-deficiency leads to improved metabolic control in obesity are not known. Our studies in the dietary obesity model have suggested that lack of obesity-induced TNFα production in adipose tissue might be part of this improved response (22). However, in the more severe obesity model, our preliminary studies did not demonstrate a significant reduction in TNFα expression in ob/ob-aP2$^{-/-}$ animals suggesting that there are additional pathways modified by the absence of aP2 leading to improved insulin sensitivity (data not shown). Because aP2 binds to cytosolic fatty acids and could modulate their intracellular concentrations, availability or subcellular trafficking, we have also postulated that in the absence of this fatty acid binding activity, the activity of nuclear hormone receptors that are regulated by fatty acid ligands could be altered. Because the PPAR family of nuclear hormone receptors play a dramatic role in glucose and lipid metabolism, they will be the prime candidates for further examination. Although our preliminary analysis have not yet yielded support for this hypothesis, further studies will be necessary to definitively address this possibility.

In any case, data presented here further support the concept of an adipopancreatic axis, the function of which is specifically altered in the absence of adipocyte aP2. How aP2-deficiency leads to reduction in lipolysis-associated insulin secretion and provides some protection from deterioration of glucose-stimulated insulin secretion in the context of obesity remains to be shown. For the development of the latter pathological state, chronic elevation of systemic FFA has been proposed as a causal signal (14). In this study, we have seen a small but consistent increase in basal plasma FFA levels in both lean and obese aP2$^{-/-}$ mice, which argues against a potential role for these molecules. On the other hand, lipolytic response is reduced, and the temporal and spatial dynamics of FFA output might still be altered and play a critical role for this physiological state. It is also possible that although small in magnitude, the elevation in basal FFA levels is sufficient to insensitize the islets to further lipid-derived stimuli. In any case, it is clear that the phenotype of the aP2$^{-/-}$ mice makes these animals a suitable model to study the interaction between adipocytes and the islets and qualifies aP2 as a very interesting drug target for the treatment of insulin resistance, dyslipidemia, and diabetes.

References

1. **Mokdad AH, Serdula MK, Dietz WH, Bowman BA, Marks JS, Koplan JP** 1999 The spread of the obesity epidemic in the United States, 1991–1998. JAMA 282:1519–1522
2. **Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH** 1999 The disease burden associated with overweight and obesity. JAMA 282:1523–1529
3. **Reaven GM** 1991 Insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypertension. Parallels between human disease and rodent models. Diabetes Care 14:195–202
4. **Nishina PM, Lowe S, Wang J, Paigen B** 1994 Characterization of plasma lipids in genetically obese mice: the mutants obese, diabetes, fat, tubby, and lethal yellow. Metab Clin Exp 43:549–553
5. **Harris MI, Hadden WC, Knowler WC, Bennett PH** 1987 Prevalence of diabetes and impaired glucose tolerance and plasma glucose levels in U.S. population aged 20–74 yr. Diabetes 36:523–534
6. **Martin BC, Warram JH, Krolewski AS, Bergman RN, Soeldner JS, Kahn CR** 1992 Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. Lancet 340:925–929
7. **Weyer C, Bogardus C, Mott DM, Pratley RE** 1999 The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. J Clin Invest 104:787–794
8. **Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI** 1999 Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. J Clin Invest 103:253–259
9. **Ferrannini E, Barrett EJ, Bevilacqua S, DeFronzo RA** 1983 Effect of fatty acids on glucose production and utilization in man. J Clin Invest 72:1737–1747
10. **Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL,Cline GW, Shulman GI** 1996 Mechanism of free fatty acid-induced insulin resistance in humans. J Clin Invest 97:2859–2865
11. **Goberna R, Tamarit JR J, Osorio J, Fussganger R,Tamarit J, Pfeiffer EF** 1974 Action of B-hydroxy butyrate, acetoacetate and palmitate on the insulin release in the perfused isolated rat pancreas. Horm Metab Res 6:256–260
12. **Crespin SR, Greenough 3rd WB, Steinberg D** 1973 Stimulation of insulin secretion by long-chain free fatty acids. A direct pancreatic effect. J Clin Invest 52:1979–1984
13. **McGarry JD, Dobbins RL** 1999 Fatty acids, lipotoxicity and insulin secretion. Diabetologia 42:128–138
14. **Sako Y, Grill VE** 1990 A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. Endocrinology 127:1580–1589
15. **Mason TM, Goh T, Tchipashvili V, Sandhu H, Gupta N, Lewis GF, Giacca A** 1999 Prolonged elevation of plasma free fatty acids desensitizes the insulin secretory response to glucose in vivo in rats. Diabetes 48:524–530
16. **Unger RH** 1995 Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. Diabetes 44:863–870
17. **Prentki M, Corkey BE** 1996 Are the β-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? Diabetes 45:273–283
18. **Boden G, Chen X** 1999 Effects of fatty acids and ketone bodies on basal insulin secretion in type 2 diabetes. Diabetes 48:577–583
19. **Coe NR, Bernlohr DA** 1998 Physiological properties and functions of intracellular fatty acid-binding proteins. Biochim Biophys Acta 1391:287–306
20. **Bernlohr DA, Doering TL, Kelly Jr TJ, Lane MD** 1985 Tissue specific expression of p422 protein, a putative lipid carrier, in mouse adipocytes. Biochem Biophys Res Commun 132:850–855
21. **Hunt CR, Ro JH, Dobson DE, Min HY, Spiegelman BM** 1986 Adipocyte P2 gene: developmental expression and homology of 5′-flanking sequences among fat cell-specific genes. Proc Natl Acad Sci USA 83:3786–3790
22. **Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM** 1996 Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. Science 274:1377–1379
23. **Scheja L, Makowski L, Uysal KT, Wiesbrock SM, Shimshek DR, Meyers DS, Morgan M, Parker RA, Hotamisligil GS** 1999 Altered insulin secretion associated with reduced lipolytic efficiency in aP2−/− mice. Diabetes 48:1987–1994
24. **Coe NR, Simpson MA, Bernlohr DA** 1999 Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels. J Lipid Res 40:967–972
25. **Randle PJ, Kerbey AL, Espinal J** 1988 Mechanisms decreasing glucose oxidation in diabetes and starvation: role of lipid fuels and hormones. Diabetes Metab Rev 4:623–638
26. **Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD** 1988 Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. Diabetes 37:1020–1024
27. **Davoren PM, Kelly W, Gries FA, Hubinger A, Whately-Smith C, Alberti KG** 1998 Long-term effects of a sustained-release preparation of acipimox on dyslipidemia and glucose metabolism in non-insulin-dependent diabetes mellitus. Metab Clin Exp 47:250–256
28. **Fulcher GR, Walker M, Farrer M, Johnson AS, Alberti KG** 1993 Acipimox increases glucose disposal in normal man independent of changes in plasma nonesterified fatty acid concentration and whole-body lipid oxidation rate. Metab Clin Exp 42:308–314
29. **Bloom JD, Dutia MD, Johnson BD, Wissner A, Burns MG, Largis EE, Dolan JA, Claus TH** 1992 Disodium (R,R)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino] propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL 316,243). A potent β-adrenergic agonist virtually specific for β3 receptors. A promising antidiabetic and antiobesity agent. J Med Chem 35:3081–3084
30. **Lafontan M, Berlan M** 1993 Fat cell adrenergic receptors and the control of white and brown fat cell function. J Lipid Res 34:1057–1091
31. **Nahmias C, Blin N, Elalouf JM, Mattei MG, Strosberg AD, Emorine LJ** 1991 Molecular characterization of the mouse β3-adrenergic receptor: relationship with the atypical receptor of adipocytes. EMBO J 10:3721–3727
32. **Grujic D, Susulic VS, Harper ME, Himms-Hagen J, Cunningham BA, Corkey BE, Lowell BB** 1997 β3-adrenergic receptors on white and brown adipocytes

mediate beta3-selective agonist-induced effects on energy expenditure, insulin secretion, and food intake. A study using transgenic and gene knockout mice. J Biol Chem 272:17686–17693
33. Yoshida T 1992 The antidiabetic β3-adrenoceptor agonist BRL 26830A works by release of endogenous insulin. Am J Clin Nutr 55:237S–241S
34. Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR 1998 A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. Mol Cell 2:559–569
35. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR 1999 Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 96:329–339
36. Lauro D, Kido Y, Castle AL, Zarnowski MJ, Hayashi H, Ebina Y, Accili D 1998 Impaired glucose tolerance in mice with a targeted impairment of insulin action in muscle and adipose tissue. Nat Genet 20:294–298
37. Taylor SI 1999 Deconstructing type 2 diabetes. Cell 97:9–12
38. Kahn BB, Rossetti L 1998 Type 2 diabetes-who is conducting the orchestra? Nat Genet 20:223–225
39. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs H, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF 1998 Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391:900–904
40. Pelton PD, Zhou L, Demarest KT, Burris TP 1999 PPARγ activation induces the expression of the adipocyte fatty acid binding protein gene in human monocytes. Biochem Biophys Res Commun 261:456–458
41. Shen WJ, Sridhar K, Bernlohr DA, Kraemer FB 1999 Interaction of rat hormone-sensitive lipase with adipocyte lipid-binding protein. Proc Natl Acad Sci USA 96:5528–5532

Lack of macrophage fatty-acid–binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis

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The adipocyte fatty-acid–binding protein, aP2, has an important role in regulating systemic insulin resistance and lipid metabolism. Here we demonstrate that aP2 is also expressed in macrophages, has a significant role in their biological responses and contributes to the development of atherosclerosis. Apolipoprotein E (ApoE)-deficient mice also deficient for aP2 showed protection from atherosclerosis in the absence of significant differences in serum lipids or insulin sensitivity. aP2-deficient macrophages showed alterations in inflammatory cytokine production and a reduced ability to accumulate cholesterol esters when exposed to modified lipoproteins. *Apoe*$^{-/-}$ mice with *Ap2*$^{+/+}$ adipocytes and *Ap2*$^{-/-}$ macrophages generated by bone-marrow transplantation showed a comparable reduction in atherosclerotic lesions to those with total aP2 deficiency, indicating an independent role for macrophage aP2 in atherogenesis. Through its distinct actions in adipocytes and macrophages, aP2 provides a link between features of the metabolic syndrome and could be a new therapeutic target for the prevention of atherosclerosis.

Adipocyte fatty-acid–binding protein, aP2 (encoded by *Ap2*), is a member of the intracellular fatty-acid–binding protein (FABP) family. Cytoplasmic FABPs are small proteins that are expressed in a highly tissue-specific manner and bind to fatty acids[1,2]. aP2 is primarily detected in adipose tissue and its expression is highly regulated during differentiation of adipocytes[3,4]. Moreover, expression of *Ap2* mRNA is transcriptionally controlled by fatty acids[5,6]. Recent studies in aP2-deficient mice have shown that loss of this protein has a critical impact on several aspects of the metabolic syndrome. First, lack of aP2 provides significant protection from hyperinsulinemia and insulin resistance associated with dietary or genetic obesity[7,8]. Second, aP2 contributes to improved systemic glucose and lipid metabolism in the setting of dietary or genetic obesity[7,8] and alters the rate of adipocyte lipolysis[9,10]. As both insulin resistance and abnormal lipid metabolism are risk factors for cardiovascular disease, it is possible that aP2 influences the development of atherosclerosis by modulating these factors.

Several lines of evidence indicate a striking overlap between the biology of adipocytes and macrophages. Genes that are critical in adipocytes, including those encoding transcription factors, cytokines, inflammatory molecules, fatty acid transporters and scavenger receptors, are also expressed in macrophages and have an important role in their biology[11]. For example, peroxisome-proliferator activated receptor-γ (PPAR-γ) is a member of the nuclear-receptor superfamily of ligand-activated transcription factors that regulates adipocyte development and glucose homeostasis. PPAR-γ is also expressed in activated monocyte/macrophages and might have a role in generating the inflammatory response and forming the foam cells associated with atherosclerotic lesions[12–16]; however, the mechanisms for these functions are unclear. Interestingly, aP2 expression was recently revealed in human monocytes following stimulation with PPAR-γ activators[17], and oxidized low-density lipoprotein has been reported to induce expression of aP2 in human THP-1 macrophages[18]. These observations indicate that expression of aP2 by macrophages might also influence foam-cell formation and thereby atherosclerotic processes, possibly through mechanisms independent of its metabolic effects.

Here we examined whether macrophage aP2 influences foam-cell formation and the development of atherosclerosis independent of the effects of aP2 on insulin resistance and plasma lipids. Using two different models, *Apoe*$^{-/-}$ mice intercrossed with *Ap2*$^{-/-}$ mice and the bone-marrow transplantation (BMT) model in which *Ap2*$^{-/-}$*Apoe*$^{-/-}$ mice are donors to recipient *Ap2*$^{+/+}$*Apoe*$^{-/-}$ mice, we demonstrate that aP2-deficient macrophages provide significant protection against atherosclerosis in the ApoE-deficient model in the absence of differences in glucose and lipid metabolism.

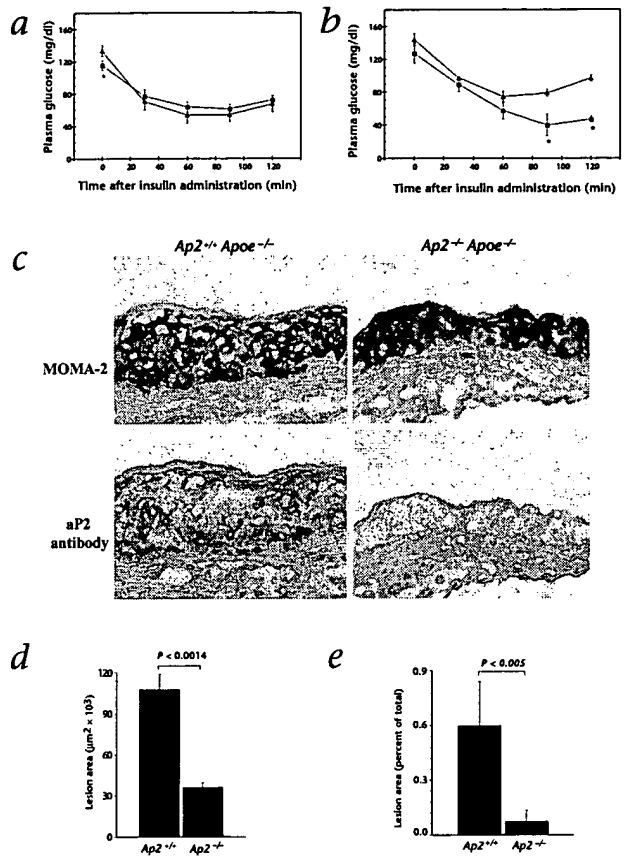

Fig. 1 Insulin sensitivity and atherosclerosis in $Ap2^{+/+}Apoe^{-/-}$ and $Ap2^{-/-}Apoe^{-/-}$ mice on chow diet. ***a*** and ***b***, Insulin tolerance tests were performed in 15 male (*a*) and 7 female (*b*) 14-week-old $Apoe^{-/-}$ (△) and $Ap2^{-/-}Apoe^{-/-}$ (■), mice. Data are mean ± s.e.m. *, $P < 0.05$. ***c***, Immunocytochemical detection of macrophages and aP2 expression in the proximal aorta of $Ap2^{+/+}Apoe^{-/-}$ (left) and $Ap2^{-/-}Apoe^{-/-}$ (right) mice. Macrophages are stained with MOMA-2 (upper), and aP2 is detected with polyclonal rabbit antiserum against mouse aP2 (lower). ***d*** and ***e***, Quantification of atherosclerotic lesion area in the proximal (*d*) and *en face* aorta (*e*) in $Ap2^{+/+}Apoe^{-/-}$ and $Ap2^{-/-}Apoe^{-/-}$ mice on chow diet. Data are represented as the average mean lesion area for each group.

Metabolism and atherosclerosis in $Ap2^{-/-}Apoe^{-/-}$ mice

Feeding C57BL/6 mice a high-fat diet promotes the development of obesity and insulin resistance[19], which could impact the development of atherosclerosis[20]. We therefore initially used ApoE-deficient mice, which on a normal chow diet develop spontaneous atherosclerotic lesions that are largely independent of obesity, hyperglycemia and hyperinsulinemia. To analyze the role of aP2 in atherosclerotic lesion formation, we intercrossed $Ap2^{-/-}$ mice with ApoE-deficient mice. At 4 weeks, we divided littermates into an experimental group of $Ap2^{-/-}Apoe^{-/-}$ ($n = 7$) and a control group of $Ap2^{+/+}Apoe^{-/-}$ ($n = 7$) mice and fed them a regular chow diet (< 4.5% fat) for 14 weeks. After insulin tolerance tests and steady-state biochemical measurements, we analyzed the extent of aortic atherosclerosis at 18 weeks. After a four-hour fast, total serum cholesterol (445 ± 36 versus 478 ± 12; mg/dl ± s.e.m.) and triglyceride (142 ± 27 versus 193 ± 13; mg/dl ± s.e.m.) levels were elevated as a result of ApoE deficiency in both $Ap2^{+/+}$ and $Ap2^{-/-}$ groups, respectively, without significant differences between genotypes. In addition, serum-glucose levels did not differ significantly between the experimental and control mice (117 ± 5.9 and 126.3 ± 13; mg/dl ± s.e.m.; $P = 0.53$). There was a reduction in total cholesterol, triglyceride and glucose, but not insulin levels in the $Ap2^{-/-}Apoe^{-/-}$ group compared with $Ap2^{+/+}Apoe^{-/-}$ controls after 24 hours fasting (data not shown). Insulin tolerance studies showed that insulin sensitivity did not differ between the male $Ap2^{-/-}Apoe^{-/-}$ and $Ap2^{+/+}Apoe^{-/-}$ mice (Fig. 1*a*). We saw similar results in the females, except that insulin tolerance tests indicated a small improvement in the $Ap2^{-/-}Apoe^{-/-}$ mice compared with $Apoe^{-/-}$ controls (Fig. 1*b*). These data showed that except for a small change in insulin sensitivity in the females, the steady state metabolic status of these mice was similar.

All of the mice developed moderate, fatty streak lesions, consisting predominantly of macrophage-derived foam cells, as determined by immunocytochemistry using the monoclonal antibody against mouse macrophages[21], MOMA-2 (Fig. 1*c*, upper panels). The macrophage-derived foam cells stained strongly positive for aP2 in the lesions of the $Ap2^{+/+}Apoe^{-/-}$ but not in the $Ap2^{-/-}Apoe^{-/-}$ mice (Fig. 1*c*, lower panels). Quantitative analysis of the proximal aorta revealed that the mean atherosclerotic lesion area in $Ap2^{-/-}Apoe^{-/-}$ males was reduced by 66% compared with $Ap2^{+/+}Apoe^{-/-}$ males (36,334 ± 3,492 versus 107,815 ± 11,144; μm^2/section ± s.e.m.; $P < 0.0014$; Fig. 1*d*). A similar reduction (87.6%) was found by *en face* analysis of the extent of atherosclerosis in the entire aorta in $Ap2^{-/-}Apoe^{-/-}$ males compared with $Ap2^{+/+}Apoe^{-/-}$ controls (0.60 ± 0.15 versus 0.074 ± 0.42; percent ± s.e.m.; $P < 0.005$; Fig. 1*e*). Thus, in the setting of a normal chow diet, male ApoE-deficient mice lacking aP2 were protected from the development of macrophage-derived foam cells and atherosclerosis. In a separate experiment, the extent of atherosclerosis in the proximal aorta was reduced by 35% in male and by 26% in female $Ap2^{-/-}Apoe^{-/-}$ mice fed the Western diet for 14 weeks compared with $Ap2^{+/+}Apoe^{-/-}$ controls ($P < 0.0024$ and $P < 0.006$, respectively; data not shown).

FABP expression and function in macrophages

Given that atherosclerotic lesions showed strong presence of aP2 and the differences in atherosclerosis occurred in the absence of significant metabolic alterations, macrophage aP2 expression might be responsible for the observed differences in atherosclerosis. We therefore investigated aP2 expression and regulation in primary isolated macrophages and macrophage cell lines. In both THP-1 and U-937 human monocyte/macrophage cell lines, we did not detect aP2 expression in resting cells but did so after stimulation by PMA (phorbol 13-myristate 12-acetate) to induce differentiation (Fig. 2*a* and *b*). Similarly, primary human monocytes did not express aP2 protein, but we observed high levels upon stimulation with PMA (Fig. 2*d*). In contrast, we did not detect aP2 expression by northern-blot analysis of resting or stimulated EL4 T-cell or M12 B-cell lines (data not shown). The keratinocyte FABP, mal1, which is the second isoform expressed in adipocytes, was also present in these cells and was regulated in an essentially identical manner upon PMA stimulation, although low levels were detectable in the resting state in THP-1 and U-937 cell lines (Fig. 2*a* and *b*). Expression of both aP2 and mal1 was also observed in isolated peritoneal mouse macrophages. Unlike the compensatory regulation in adipocytes, mRNA encoding mal1 did not appear to be significantly upregulated in $Ap2^{-/-}$ primary macrophages (Fig. 2*c*). We then investigated whether the regulatory elements that direct expression of aP2 in adipocytes are sufficient to confer expression in macrophages of transgenic mice. For this we used three independent lines expressing genes encoding uncoupling protein (UCP)-1,

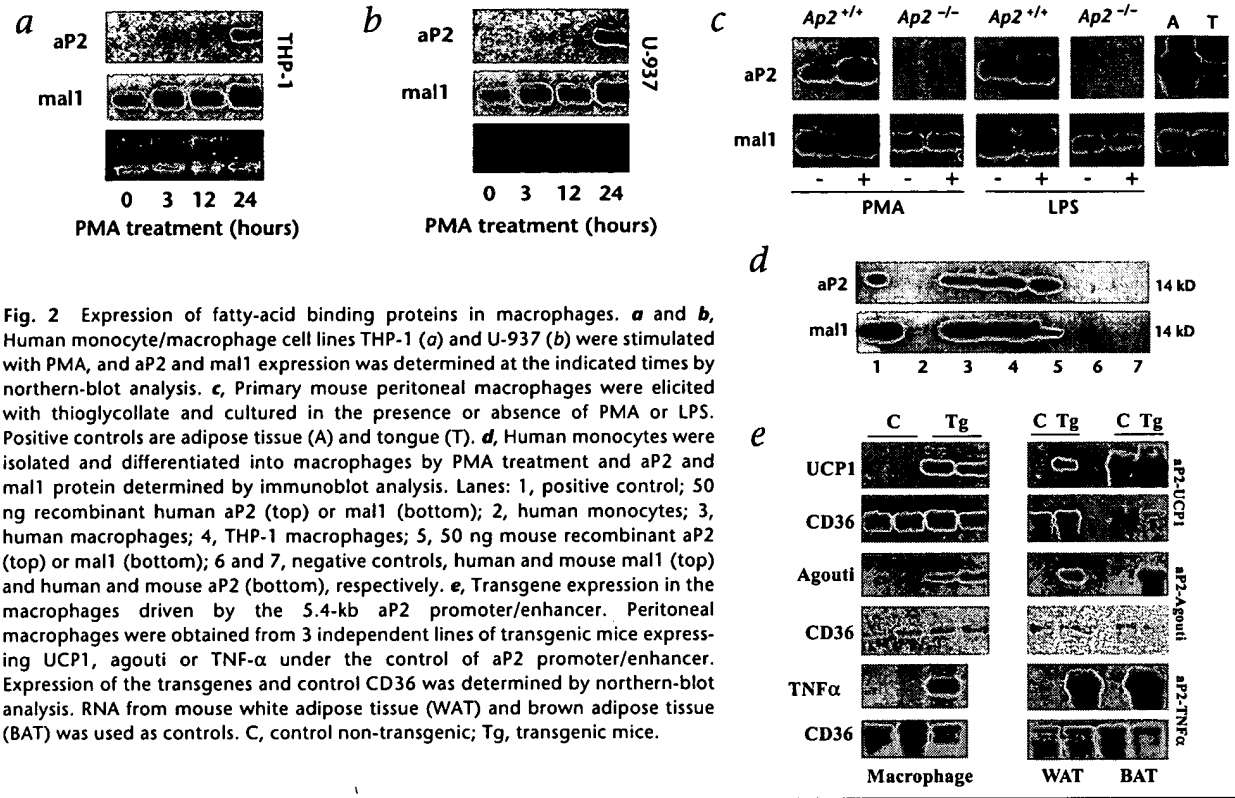

Fig. 2 Expression of fatty-acid binding proteins in macrophages. *a* and *b*, Human monocyte/macrophage cell lines THP-1 (*a*) and U-937 (*b*) were stimulated with PMA, and aP2 and mal1 expression was determined at the indicated times by northern-blot analysis. *c*, Primary mouse peritoneal macrophages were elicited with thioglycollate and cultured in the presence or absence of PMA or LPS. Positive controls are adipose tissue (A) and tongue (T). *d*, Human monocytes were isolated and differentiated into macrophages by PMA treatment and aP2 and mal1 protein determined by immunoblot analysis. Lanes: 1, positive control; 50 ng recombinant human aP2 (top) or mal1 (bottom); 2, human monocytes; 3, human macrophages; 4, THP-1 macrophages; 5, 50 ng mouse recombinant aP2 (top) or mal1 (bottom); 6 and 7, negative controls, human and mouse mal1 (top) and human and mouse aP2 (bottom), respectively. *e*, Transgene expression in the macrophages driven by the 5.4-kb aP2 promoter/enhancer. Peritoneal macrophages were obtained from 3 independent lines of transgenic mice expressing UCP1, agouti or TNF-α under the control of aP2 promoter/enhancer. Expression of the transgenes and control CD36 was determined by northern-blot analysis. RNA from mouse white adipose tissue (WAT) and brown adipose tissue (BAT) was used as controls. C, control non-transgenic; Tg, transgenic mice.

agouti and tumor necrosis factor (TNF)-α under the control of the 5.4-kb aP2 promoter/enhancer. Primary macrophages from all three transgenic lines showed high levels of expression of these transgenes (Fig. 2*e*). The aP2 promoter/enhancer thus directs expression in macrophages as well as adipocytes.

We next investigated whether aP2 deficiency has functional consequences in macrophage biology that might be relevant to the development of atherosclerosis. We first determined the expression of several inflammatory cytokines, including TNF-α, interleukin (IL)-1β and IL-6 in *Ap2*$^{-/-}$ macrophages and wild-type controls. In the resting state, expression of these cytokines was significantly reduced in the aP2-deficient macrophage cell lines compared with *Ap2*$^{+/+}$ controls (Fig. 3*a*). Moreover, whereas an increase in TNF-α, IL-1β, and IL-6 expression was evident in the wild-type cells upon PMA stimulation, we observed no changes in the *Ap2*$^{-/-}$ cells, demonstrating a reduced inflammatory capacity of *Ap2*$^{-/-}$ macrophages (Fig. 3*a*).

To examine the potential function of aP2 in macrophage lipid deposition, cholesterol ester accumulation was assayed in acetylated low-density lipoprotein (Ac-LDL)-loaded macrophages derived from wild-type and *Ap2*$^{-/-}$ mice. Lipids extracted from cells were assayed using a Nile Red fluorescent-lipid thin-layer chromatography (TLC). *Ap2*$^{-/-}$ macrophages responded to Ac-LDL loading with 37% less accumulation of cholesterol ester compared with the *Ap2*$^{+/+}$ controls ($P < 0.001$; Fig. 3*b*). In the absence of lipid loading, the cholesterol ester levels in the *Ap2*$^{-/-}$ cells were 29% lower than in *Ap2*$^{+/+}$ cells ($P < 0.01$). We also evaluated the levels of aP2 and mal1 protein expression in the *Ap2*$^{-/-}$ and wild-type macrophages. Under the conditions of the lipid accumulation studies, the aP2 protein level in the wild-type line (2 ng/μg cell protein) was approximately 22% that of mal1 (9 ng/μg). In the *Ap2*$^{-/-}$ line, aP2 was not detectable and mal1 was 20.9 ng/μg. Exposure to Ac-LDL did not affect the expression levels of either

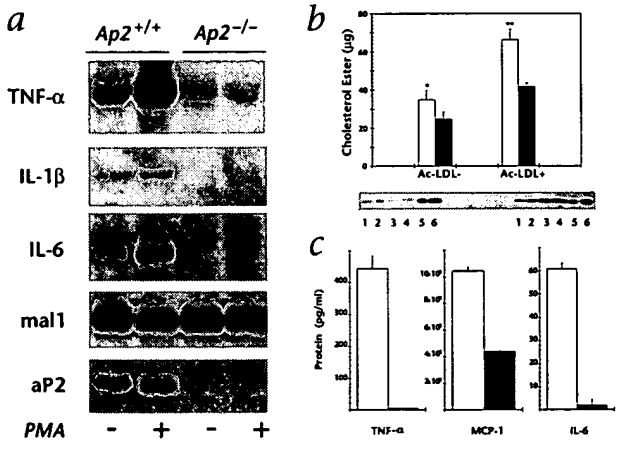

Fig. 3 Expression of inflammatory cytokines and cholesterol ester levels in *Ap2*$^{-/-}$ macrophages. *a*, TNF-α, IL-1β and IL-6 mRNA levels were determined following PMA treatment. The bottom two blots show mal1 and aP2 mRNA expression. *b* and *c*, Accumulation of cholesterol esters (*b*) and cytokine secretion (*c*) in control (□) and *Ap2*$^{-/-}$ (■) macrophages. Cholesterol ester levels were determined before and after treatment with Ac-LDL. Cytokine levels were determined by ELISA in the conditioned medium following treatment with Ac-LDL. The graph shows mean ± s.e. from 10 independent experiments. Immunoblot analysis of aP2 (L) and mal1 (R) protein expression is shown in *b*, lower. Lanes: 1, *Ap2*$^{+/+}$ without Ac-LDL; 2, *Ap2*$^{+/+}$ with Ac-LDL; 3, *Ap2*$^{-/-}$ without Ac-LDL; 4, *Ap2*$^{-/-}$ with Ac-LDL; 5&6, 20 and 40 ng of recombinant murine aP2 (left) or Mal1 (right) standards.

Table 1 Serum lipids and glucose in $AP2^{-/-}$ BMT mice

Group	Serum study	Baseline	4 wk post-BMT	8 wk post-BMT	13 wk post-BMT
$Ap2^{+/+}Apoe^{-/-}\rightarrow Apoe^{-/-}$ (male), $n = 8$	Cholesterol	350 ± 46	416 ± 80	423 ± 52	448 ± 109
	Triglycerides	147 ± 36	169 ± 54	120 ± 39	137 ± 49
	Glucose			110 ± 18	127 ± 39
$Ap2^{-/-}Apoe^{-/-}\rightarrow Apoe^{-/-}$ (male), $n = 9$	Cholesterol	331 ± 43	450 ± 37	371 ± 66	395 ± 88
	Triglycerides	135 ± 40	144 ± 50	129 ± 32	135 ± 31
	Glucose			110 ± 9	135 ± 27
$Ap2^{+/+}Apoe^{-/-}\rightarrow Apoe^{-/-}$ (female), $n = 11$	Cholesterol	282 ± 64	406 ± 67	454 ± 56	300 ± 45
	Triglycerides	63 ± 15	69 ± 19	81 ± 27	98 ± 46
	Glucose			101 ± 31	94 ± 18
$Ap2^{-/-}Apoe^{-/-}\rightarrow Apoe^{-/-}$ (female), $n = 8$	Cholesterol	265 ± 59	443 ± 85	432 ± 110	283 ± 92
	Triglycerides	53 ± 12	80 ± 16	85 ± 22	118 ± 48
	Glucose			90 ± 42	107 ± 14

Fasting (4 h) total serum-cholesterol, -triglyceride and -glucose levels in male and female $Apoe^{-/-}$ mice before and after BMT with $Ap2^{+/+}Apoe^{-/-}$ or $Ap2^{-/-}Apoe^{-/-}$ marrow. Values (mg/dL) are mean ± s.d.

FABP in this model (Fig. 3*b*, lower panel). These results indicate that genetic elimination of aP2 significantly decreased intracellular cholesterol ester accumulation in macrophages despite the presence of substantial amounts of mal1 protein. We also determined cytokine production from these cells under the same experimental conditions. These experiments demonstrated a strong reduction in the capacity of $Ap2^{-/-}$ cells to secrete TNF-α, monocyte chemotactic protein (MCP)-1, and IL-6 proteins compared with wild-type controls (Fig. 3*c*).

Bone-marrow transplantation studies

BMT studies in gene-targeted mice have been a powerful tool to examine the contribution of various genes expressed by macrophages to foam-cell formation and atherogenesis[22–25]. We therefore created $Apoe^{-/-}$ mice chimeric for macrophage aP2 expression using BMT to determine the contribution of macrophage aP2-deficiency in atherogenesis. Ten-week-old male $Apoe^{-/-}$ mice were lethally radiated (9 Gy) and transplanted with either $Ap2^{-/-}Apoe^{-/-}$ (experimental group, $n = 10$) or $Ap2^{+/+}Apoe^{-/-}$ (control group, $n = 8$) bone marrow. Similarly, 10-week-old female $Apoe^{-/-}$ mice were lethally radiated and transplanted with either $Ap2^{-/-}Apoe^{-/-}$ (experimental group, $n = 8$) or $Ap2^{+/+}Apoe^{-/-}$ (control group, $n = 11$) bone marrow. The transplanted mice were fed a standard chow diet (< 4.5% fat) for 13 weeks. We observed no significant differences in serum levels of glucose, cholesterol or triglycerides between the experimental and control groups in either experiment at 8 or 13 weeks after transplantation (Table 1). Moreover, the distribution of cholesterol among the serum lipoproteins as determined by size exclusion chromatography did not differ significantly between the experimental and control groups in either experiment (Fig. 4*a*). Finally, insulin tolerance tests showed that insulin sensitivity did not differ between the two groups (data not shown). Therefore, the lack of macrophage aP2 expression had no impact on fasting serum-glucose and -lipid levels or lipoprotein profiles under these dietary conditions.

Thirteen weeks after transplantation, the extent of atherosclerosis in the proximal aortas of the transplanted $Apoe^{-/-}$ mice was determined. Staining of cross-sections of the proximal aorta with Oil Red O revealed fatty streak lesions, which in the males consisted almost exclusively of macrophage-derived foam cells, as determined by immunocytochemical staining with MOMA-2 (Fig. 4*b*). Immunocytochemical analysis of serial sections of the proximal aorta by staining with MOMA-2 for mouse macrophages (Fig. 4*b*, upper panels), or the aP2 protein (Fig. 4*b*, lower panels) revealed

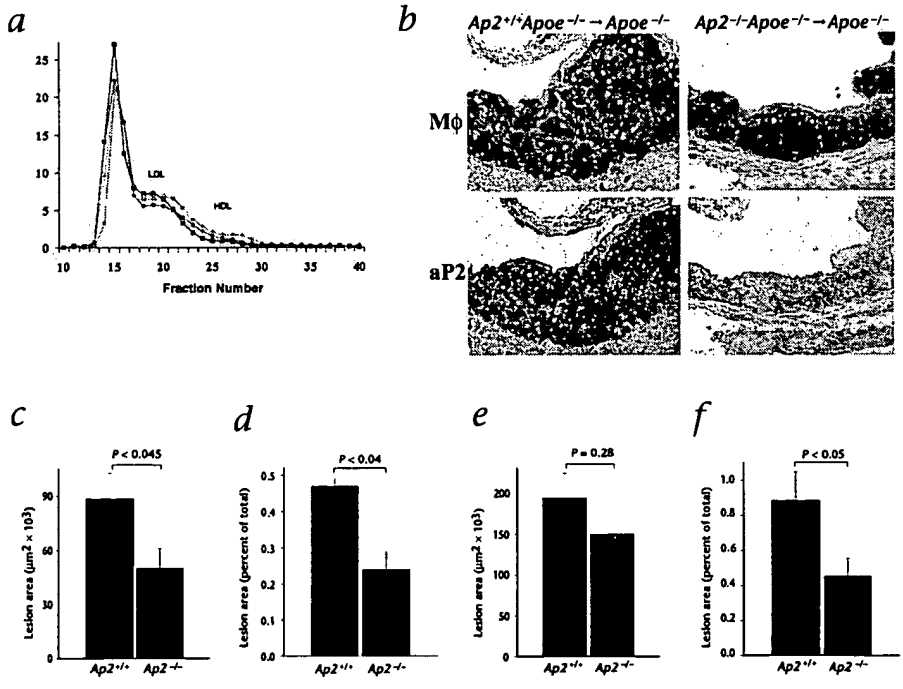

Fig. 4 Lipoprotein distribution, immunochemistry and atherosclerosis in $Ap2^{-/-}$ BMT mice. ***a***, Lipoprotein distribution in $Apoe^{-/-}$ mice with transplanted marrow after 13 wk on standard chow diet. ▨, female $Ap2^{-/-}Apoe^{-/-}$; ■, female $Ap2^{+/+}Apoe^{-/-}$; ■, male $Ap2^{-/-}Apoe^{-/-}$; ■, male $Ap2^{+/+}Apoe^{-/-}$. Data are represented as an average (n = 3) percent distribution of total cholesterol for each group. Fractions 14–17 contain VLDL; fractions 18–24 are IDL/LDL; and fractions 25–29 contain HDL. Fractions 30–40 are the non–lipoprotein-associated proteins. ***b***, Immunocytochemical detection of macrophages and aP2 expression in the proximal aorta of $Apoe^{-/-}$ mice transplanted with $Ap2^{+/+}Apoe^{-/-}$ (left) or $Ap2^{-/-}Apoe^{-/-}$ (right) marrow. Macrophages are stained as in Fig. 1*c*. ***c–f***, Quantification of atherosclerotic lesion area in the proximal and the en face aorta, respectively for male (*c* and *d*) and female (*e* and *f*) $Apoe^{-/-}$ mice 13 weeks after receiving $Ap2^{+/+}Apoe^{-/-}$ or $Ap2^{-/-}Apoe^{-/-}$ marrow. The atherosclerotic lesions were stained and quantified as noted in Fig. 1. Data are represented as the average mean lesion area for each group.

that macrophage-derived foam cells in $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice colocalized with aP2 protein. Macrophages from $Ap2^{-/-} \rightarrow Apoe^{-/-}$ mice did not react with the aP2 antibody. Quantitative analysis of the extent of atherosclerotic lesions in the proximal aorta revealed that mean lesion area in male $Ap2^{-/-} \rightarrow Apoe^{-/-}$ mice was reduced by 43% compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice (50,311 ± 10,600 and 88,688 ± 13,950; μm²/section ± s.e.m., respectively; $P < 0.045$; Fig. 4*c*). *En face* analysis of the extent of atherosclerosis in the entire aorta in $Ap2^{-/-} \rightarrow Apoe^{-/-}$ males revealed a 48% reduction compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ males (0.24 ± 0.05 versus 0.47 ± 0.095; precent ± s.e.m.; $P < 0.04$; Fig. 4*d*). Thus, male $Apoe^{-/-}$ mice reconstituted with $Ap2^{-/-}$ macrophages are protected from atherosclerosis compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice. As expected, the extent of atherosclerosis was greater in the female ApoE transplant recipients, and lesions in their proximal aorta were significantly more advanced, showing the presence of smooth muscle cells in the intima. Quantitative analysis of the extent of atherosclerotic lesions in the proximal aorta revealed a trend for a reduction in mean lesion area in female $Ap2^{-/-} \rightarrow Apoe^{-/-}$ mice compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice (150,074 ± 23,347 and 194,358 ± 29,798; μm²/section ± s.e.m., respectively; $P = 0.288$; Fig. 4*e*). However, *en face* analysis of the extent of atherosclerosis in the entire aorta in $Ap2^{-/-} \rightarrow Apoe^{-/-}$ females revealed a significant 48% reduction in mean lesion area compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice (0.46 ± 0.05 versus 0.89 ± 0.16, percent ± s.e.m.; $P < 0.04$; Fig. 4*f*). These transplantation studies demonstrate a role for macrophage aP2 expression in promoting foam-cell formation and atherogenesis *in vivo*.

Discussion

Previous studies have demonstrated that deficiency of aP2, a downstream target of PPAR-γ, predominantly affects adipocytes and contributes to improved systemic glucose and lipid metabolism in the setting of dietary or genetic obesity[7,8]. Here, we demonstrate that aP2 is also strongly expressed in macrophages and modulates their biological responses. Total and macrophage-specific aP2 deficiencies led to a striking protection from the development of atherosclerosis in the $Apoe^{-/-}$ model. Based on these data, a potential mechanism underlying the metabolic syndrome emerges from coordinated modulation of the metabolic status of adipocytes and the metabolic or inflammatory status of macrophages through pathways common to both cell types.

The gene encoding aP2 is expressed at high levels in mouse and human macrophages upon activation *in vitro* and in macrophages associated with atherosclerotic lesions *in vivo*, raising the possibility that it might impact the macrophage inflammatory response and foam-cell formation. As in adipocytes, production of TNF-α is greatly reduced in $Ap2^{-/-}$ macrophages compared with wild-type controls[7]. Other inflammatory cytokines such as IL-1β and IL-6 are also suppressed in $Ap2^{-/-}$ macrophages. Moreover, $Ap2^{-/-}$ macrophages display significantly decreased intracellular cholesterol ester accumulation *in vitro* and secrete highly reduced quantities of TNF-α, MCP-1 and IL-6 upon exposure to modified lipoprotein. These results indicate that aP2 has a significant role in two important aspects of macrophage biology that are highly relevant to the pathogenesis of atherosclerosis. mal1, a close relative of aP2, presents an expression pattern similar to aP2. However, whereas the expression of mal1 mRNA in $Ap2^{-/-}$ adipocytes is greatly enhanced, compensatory regulation in $Ap2^{-/-}$ macrophages is mild. Hence, it will be important to determine the impact of mal1 and combined aP2/mal1 deficiency in metabolic regulation and atherosclerosis. Finally, macrophage aP2 expression is controlled with the same promoter/enhancer elements that confer expression in adipocytes; this might have important implications in the assessment of many transgenic lines generated with the 5.4-kb aP2 promoter/enhancer.

Both macrophages and adipocytes are sites for active lipid metabolism and signaling. Therefore, it is not surprising that FABPs have a strong impact on the biology of these cells. This is likely to involve many aspects of lipid metabolism in both cell types including transport of lipids and shuttling them to specific metabolic or signaling pathways. Recent studies with other isoforms have indicated potential regulation of nuclear hormone receptor activity in the liver through direct transportation of lipid and xenobiotic ligands[26]. Regulation of macrophage ACAT activity[27–29] or the availability of FA for esterification with cholesterol might also be altered by FABPs. Alternatively, FABPs could impact the rates of transport through quantitative or functional alterations in fatty-acid transporters or scavenger receptors. Further studies will be required to address these possibilities.

A critical question is whether the function of aP2 in the macrophage is sufficient to modulate development of atherosclerosis in a way that is distinguishable from the metabolic consequences of aP2 deficiency. We have taken several steps to address this question. First, we studied the effect of aP2 deficiency on the development of atherosclerosis in $Apoe^{-/-}$ mice on normal chow diet. On this diet, ApoE-deficient mice exhibit severe hypercholesterolemia and spontaneous atherosclerotic lesions in the absence of significant changes in body weight or serum levels of glucose or insulin. Under these conditions, male $Ap2^{-/-}Apoe^{-/-}$ mice, despite severe hypercholesterolemia, developed 66% less atherosclerosis in cross-sections of the proximal aorta and 86% smaller lesions by *en face* analysis of the extent of lesions in the entire aorta compared with control $Ap2^{+/+}Apoe^{-/-}$ mice. These reductions in the extent of atherosclerosis occurred in the absence of significant changes in serum-lipid, -glucose or -insulin levels, supporting the hypothesis that aP2 expression contributes to atherosclerosis independent of its established role in glucose and lipid metabolism.

Second, we generated macrophage-restricted aP2 deficiency through BMT experiments to examine the hypothesis that macrophage aP2 expression promotes atherogenesis independent of its expression by adipocytes. Male $Apoe^{-/-}$ mice reconstituted with $Ap2^{-/-}$ macrophages had a 43% and 49% reduction in lesion area compared with those reconstituted with $Ap2^{+/+}$ cells, as assessed by both lesion size in cross sections of the proximal aorta and in the entire aorta *en face*, respectively. As expected, the extent of atherosclerosis was greater in the proximal aortas of female ApoE-deficient transplanted mice than in the males, and the reduction in vascular lesions in female $Ap2^{-/-}Apoe^{-/-} \rightarrow Apoe^{-/-}$ mice at this site was not statistically significant. Although this observation indicates a potential gender difference regarding the effect of aP2 expression in atherosclerosis, we think that it most likely reflects the fact that the lesions in the proximal aorta of the females were more advanced. These include intermediate lesions with smooth muscle cell involvement, indicating that the contribution of macrophage aP2 expression might be lessened in the setting of more advanced lesions. In the whole aorta, a significant 48% reduction in mean lesion area was evident in female $Ap2^{-/-}Apoe^{-/-} \rightarrow Apoe^{-/-}$ mice compared with female $Ap2^{+/+}Apoe^{-/-} \rightarrow Apoe^{-/-}$ mice. This may be explained by the fact that mice develop atherosclerotic lesions first in the proximal aorta and the lesions progress distally, resulting in more advanced lesions in the proximal than the distal aorta[30]. Moreover, in response to 14 weeks on the Western-type diet, female $Apoe^{-/-}Ap2^{-/-}$ mice show reduced atherosclerosis in the proximal aorta compared with $Apoe^{-/-}Ap2^{-/-}$ con-

trols, indicating that the effects of aP2 on atherosclerosis are not gender specific. These results demonstrate a significant role for macrophage aP2 in atherogenesis independent of its expression in adipocytes and its role in glucose and lipid metabolism.

Taken together with the previous observations regarding the impact of aP2 on glucose and lipid metabolism, our data support an important role for aP2 in understanding the molecular basis of metabolic syndrome via its coordinated action on both metabolic and inflammatory responses. These findings should provide further insights into the molecular mechanisms leading to these diseases and generate opportunities for the development of a novel class of therapeutic modalities to treat them.

Methods

Animal procedures. Mice homozygous for inactivation of *Ap2* were backcrossed 12 generations into the C57BL/6 background[8,10]. These $Ap2^{-/-}$ mice were intercrossed with the ApoE-deficient mice also in the C57BL/6 background to generate mice heterozygous at both loci. These $Ap2^{-/+}Apoe^{-/+}$ mice were then intercrossed to produce $Ap2^{-/-}Apoe^{-/-}$ mice along with $Ap2^{+/+}Apoe^{-/-}$ littermate controls. At 4 weeks, littermates were divided into an experimental group of $Ap2^{-/-}Apoe^{-/-}$ and a control group of $Ap2^{+/+}Apoe^{-/-}$ mice. For the BMT study, donor mice were derived from the same colony of $Ap2^{-/-}Apoe^{-/-}$ and $Ap2^{+/+}Apoe^{-/-}$ mice described above. Recipient mice were derived from an established colony at our mouse facility of ApoE-deficient mice on the C57BL/6 background[22,31]. Mice were fed standard chow with 4.5% fat (PMI Feeds, St. Louis, Missouri) or Western diet (diet #TD88137, Harland Teklad, Madison, Wisconson). UCP1 and agouti transgenic mice were provided by L. Kozak. Transgenic mice expressing a membrane-bound form of TNF-α driven by the aP2 promoter/enhancer in the TNF-α–deficient background were generated in one of our laboratories (Harvard). Animal care and experimental procedures were performed under approval from the Animal Care Committees of Vanderbilt and Harvard Universities.

Bone-marrow transplantation. 1 week before and 2 weeks following BMT, 100 mg/L neomycin and 10 mg/L polymyxin B sulfate (Sigma) were added to the acidified water. Bone marrow was collected from donor mice by flushing femurs with RPMI 1640 media (GIBCO, Grand Island, New York) containing 2% FBS and 5 U/ml heparin (Sigma). Recipient mice were lethally irradiated (9 Gy) by a cesium gamma source. 4 hours later, 5–10 × 10^6 bone-marrow cells in 0.3 ml were transplanted by tail-vein injection as described[22,32].

Serum measurements and insulin tolerance tests. Mice were fasted for either 4 h during day or 24 h overnight and blood samples were collected by retro-orbital venous plexus puncture under isoflurane anesthesia (IsoVet, Schering-Plough, Union, New Jersey). Serum was separated by centrifugation and 1 mM phenylmethylsulfonyl fluoride was added (Sigma). The serum total cholesterol and triglycerides were determined using Sigma kit #352 and kit #339 adapted for microtiter plate assay as described[33]. Blood glucose concentrations were determined on 5 µl whole blood by using gluco-analyser blood glucose strips (Medisense, Bedford, Massachussetts). Steady-state insulin concentrations were determined with a commercially available radioimmunoassay (Linco, St. Charles, Missouri). Lipoprotein assays were performed as described[30]. Insulin tolerance tests (0.5 IU/kg) were performed on conscious mice following a 6-h fast as described[7,34,35].

Immunocytochemistry and quantification of arterial lesions. To detect macrophages and the aP2 protein in arterial lesions, 5-µm serial cryosections of the proximal aorta were incubated with either a polyclonal rabbit antiserum against mouse aP2 (gift of D. Bernlohr) or rat antibody against mouse macrophages[21], MOMA-2 (Accurate, Westbury, New York) as described[23]. For quantification of arterial lesions, aortas were pinned out in an *en face* preparation as described[30,36]. 10-µm thick cryosections of the proximal aorta were stained with Oil Red O and counterstained with hematoxylin, as described[23,37]. Quantitative analysis of lipid-stained lesions was performed using an Imaging System KS 300 (Release 2.0; Kontron Electronik GmbH, Eden Prairie, Minnesota). Color threshold was used to delimit the Oil-Red-O–stained lesion area that was measured as squared microns per mouse.

Macrophage studies. PBMCs were prepared from healthy donors as described[38] and were incubated overnight in RPMI medium + 5% FBS to spontaneously differentiate into macrophages. THP-1 (ATCC #TIB 202) and U-937 (ATCC #CRL-1593.2) human monocytic leukemia cell lines were grown in RPMI with 5% FBS and differentiated by 25 or 100 nM PMA into macrophages. Immortalized $Ap2^{+/+}$ and $Ap2^{-/-}$ murine macrophage cell lines were generated in our laboratory by a modification of a described procedure[39]. Primary macrophages are isolated from the peritoneal cavity and were incubated with the indicated reagents for 24 hours. $Ap2^{+/+}$ and $Ap2^{-/-}$ mouse macrophage cell lines were grown to near confluence in 12-well plates in RPMI-1640 with 5% FBS. Supernatants were collected after 72 h of treatment with 50 µg/ml Ac-LDL (Biomedical Technologies, Stoughton, Massachusetts) and cytokine levels were determined by commercially available ELISA systems (TNF-α from R&D, Minneapolis, Minnesota; IL-6 and MCP-1 from PharMingen, San Diego, California). Macrophage cell lines were cultured and lipids were extracted from the cells for TLC analysis as described[40]. Immunoblots were conducted with rabbit antibodies against human recombinant aP2 and mal1 as described[10].

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1. Glatz, J.F.C. & van der Vusse, G.J. Cellular fatty acid-binding proteins:their function and physiological significance. *Prog. Lipid Res.* **35**, 243–282 (1996).
2. Coe, N.R. & Bernlohr, D.A. Physiological properties and functions of intracellular fatty acid-binding proteins. *Biochim. Biophys. Acta* **1391**, 287–306 (1998).
3. Spiegelman, B.M., Frank, M. & Green, H. Molecular cloning of mRNA from 3T3 adipocytes. Regulation of mRNA content for glycerolphosphate dehydrogenase and other differentiation-dependent proteins during adipocyte development. *J. Biol. Chem.* **258**, 10083–10089 (1983).
4. Hunt, C.R., Ro, J.H., Dobson, D.E., Min, H.Y. & Spiegelman, B.M. Adipocyte P2 gene: developmental expression and homology of 5'-flanking sequences among fat cell-specific genes. *Proc. Natl. Acad. Sci. USA* **83**, 3786–3790 (1986).
5. Amri, E.-Z., Bertrand, B., Ailhaud, G. & Grimaldi, P. Regulation of adipose cell differentiation. I. Fatty acids are inducers of the aP2 gene expression. *J. Lipid Res.* **32**, 1449–1456 (1991).
6. Distel, R.J., Robinson, G.S. & Spiegelman, B.M. Fatty acid regulation of gene expression. Transcriptional and post-transcriptional mechanisms. *J. Biol. Chem.* **267**, 5937–5941 (1992).
7. Hotamisligil, G.S. *et al.* Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* **274**, 1377–1379 (1996).
8. Uysal, K.T., Scheja, L., Wiesbrock, S.M., Bonner-Weir, S. & Hotamisligil, G.S. Improved glucose and lipid metabolism in genetically obese mice lacking aP2. *Endocrinology* **141**, 3388–3396 (2000).
9. Coe, N.R., Simpson, M.A. & Bernlohr, D.A. Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels. *J. Lipid Res.* **40**, 967–972 (1999).
10. Scheja, L. *et al.* Altered insulin secretion associated with reduced lipolytic efficiency in aP2–/– mice. *Diabetes* **48**, 1987–1994 (1999).
11. Cousin, B. *et al.* A role for preadipocytes as macrophage-like cells. *FASEB J.* **13**, 305–312 (1999).
12. Ricote, M., Li, A.C., Willson, T.M., Kelly, C.J. & Glass, C.K. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* **391**, 79–82 (1998).
13. Jiang, C., Ting, A.T. & Seed, B. PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. *Nature* **391**, 82–86 (1998).
14. Ricote, M. *et al.* Expression of the peroxisome proliferator-activated receptor γ (PPAR γ) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA* **95**, 7614–7619 (1998).
15. Nagy, L., Tontonoz, P., Alvarez, J.G., Chen, H. & Evans, R.M. Oxidized LDL regulates macrophage gene expression through ligand activation of PPAR-γ. *Cell* **93**, 229–240 (1998).
16. Tontonoz, P., Nagy, L., Alvarez, J.G.A., Thomazy, V.A. & Evans, R.M. PPAR-γ Promotes Monocyte/Macrophage Differentiation and Uptake Of Oxidized LDL. *Cell* **93**, 241–252 (1998).
17. Pelton, P.D., Zhou, L., Demarest, K.T. & Burris, T.P. PPARγ activation induces the expression of the adipocyte fatty acid binding protein gene in human monocytes. *Biochem. Biophys. Res. Comm.* **261**, 456–458 (1999).
18. Fu, Y., Luo, N. & Lopes-Virella, M.F. Oxidized LDL induces the expression of ALBP/aP2 mRNA and protein in human THP-1 macrophages. *J. Lipid Res.* **41**,

2017–2023 (2000).
19. Surwit, R.S., Kuhn, C.M., Cochrane, C., McCubbin, J.A. & Feinglos, M.N. Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* **37**, 1163–1167 (1988).
20. Kunjathoor, V.V., Wilson, D.L. & LeBoeuf, R.C. Increased atherosclerosis in streptozotocin-induced diabetic mice. *J. Clin.Invest.* **97**, 1767–1773 (1996).
21. Kraal, G., Rep, M. & Janse, M. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. *Scand. J. Immunol.* **26**, 653–661 (1987).
22. Linton, M.F., Atkinson, J.B. & Fazio, S. Prevention of atherosclerosis in apoE deficient mice by bone marrow transplantation. *Science* **267**, 1034–1037 (1995).
23. Fazio, S. *et al.* Increased atherosclerosis in C57BL/6 mice reconstituted with apolipoprotein E null macrophages. *Proc. Natl. Acad. Sci. USA* **94**, 4647–4652 (1997).
24. Linton, M.F., Babaev, V.R., Gleaves, L.A. & Fazio, S. A direct role for the macrophage low density lipoprotein receptor in atherosclerotic lesion formation. *J. Biol. Chem.* **274**, 19204–19210 (1999).
25. Babaev, V.R. *et al.* Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis *in vivo*. *J. Clin. Invest.* **103**, 1697–1705 (1999).
26. Wolfrum, C., Borrmann, C.M., Borchers, T. & Spener, F. Fatty acids and hypolipidemic drugs regulate peroxisome proliferator-activated receptors α- and γ-mediated gene expression via liver fatty acid binding protein: A signaling path to the nucleus. *Proc. Natl. Acad. Sci. USA* **98**, 2323–2328 (2001).
27. Davis, P.J. n-3 and n-6 polyunsaturated fatty acids have different effects on acyl-CoA:cholesterol acyltransferase in J774 macrophages. *Biochem. Cell Biol.* **70**, 1313–1318 (1992).
28. Rumsey, S.C., Galeano, N.F., Lipschitz, B. & Deckelbaum, R.J. Oleate and other long chain fatty acids stimulate low density lipoprotein receptor activity by enhancing acyl coenzyme A:cholesterol acyltransferase activity and altering intracellular regulatory cholesterol pools in cultured cells. *J. Biol. Chem.* **270**, 10008–10016 (1995).
29. Fazio, S. *et al.* Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J. Clin. Invest.* **107**, 163–171 (2001).
30. Babaev, V.R., Patel, M.B., Semenkovich, C.F., Fazio, S. & Linton, M.F. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in low density lipoprotein receptor-deficient mice. *J. Biol. Chem.* **275**, 26293–26299 (2000).
31. Zhang, S.H., Reddick, R.L., Piedrahita, J.A. & Maeda, N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **258**, 468–471 (1992).
32. Linton, M.F., Hasty, A.H., Babaev, V.R. & Fazio, S. Hepatic ApoE expression is required for remnant lipoprotein clearance in the absence of the low density lipoprotein receptor. *J. Clin. Invest.* **101**, 1726–1736 (1998).
33. Fazio, S. *et al.* Leukocyte low density lipoprotein receptor (LDL-R) does not contribute to LDL clearance *in vivo*: bone marrow transplantation studies in the mouse. *J. Lipid Res.* **38**, 391–400 (1997).
34. Uysal, K.T., Wiesbrock, S.M., Marino, M.W. & Hotamisligil, G.S. Protection from obesity-induced insulin resistance in mice lacking TNF-α function. *Nature* **389**, 610–614 (1997).
35. Uysal, K.T., Wiesbrock, S.M. & Hotamisligil, G.S. Functional analysis of TNF receptors in TNF-α-mediated insulin resistance in genetic obesity. *Endocrinology* **139**, 4832–4838 (1998).
36. Tangirala, R.K., Rubin, E.M. & Palinski, W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J. Lipid Res.* **36**, 2320–2328 (1995).
37. Paigen, B., Morrow, A., Holmes, P.A., Mitchell, D. & Williams, R.A. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* **68**, 231–240 (1987).
38. Kiener, P.A. *et al.* Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. *J. Immunol.* **155**, 4917–4925 (1995).
39. Blasi, E. *et al.* Selective immortalization of murine macrophages from fresh bone marrow by a raf/myc recombinant murine retrovirus. *Nature* **318**, 667–670 (1985).
40. Brown, W.J., Warfel, J. & Greenspan, P. Use of Nile red stain in the detection of cholesteryl ester accumulation in acid lipase-deficient fibroblasts. *Arch. Pathol. Lab. Med.* **112**, 295–297 (1988).